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# PROVISIONAL APPLICATION TRANSMITTAL SHEET

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Sir:

Transmitted herewith for filing is a provisional patent application under CFR 1.53(c) of Inventors:

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Title: AMINOGLYCOSIDE SUPPRESSION OF TERMINATION

Enclosed are:

- ☒ 40 pages of the application (including description and claims).
- ☐ pages Sequence Listing.
- ☒ 11 sheets of ☐ formal ☒ informal drawing(s).
- ☐ Abstract.
- ☒ 49 claims.
- ☐ Small entity status is claimed under 37 CFR 1.9(f)
- ☒ Cover Sheet.
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**PROVISIONAL  
PATENT APPLICATION**

**AMINOGLYCOSIDE SUPPRESSION OF TERMINATION**

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## AMINOGLYCOSIDE SUPPRESSION OF TERMINATION

### FIELD OF THE INVENTION

The present invention relates to various methods based on selective suppression of stop  
5 codons during protein translation using an aminoglycoside antibiotic, including methods for alternative production of soluble or membrane-bound proteins from the same cell, for selection of cell clones or cells, and for evaluation of protein expression.

### BACKGROUND OF THE INVENTION

10 While the anticodons of aminoacyl transfer RNAs (tRNAs) recognize sense codons, leading to the incorporation of a specific amino acid, there are no eukaryotic tRNAs with anticodons that match any of the three stop (nonsense) codons UAA, UGA and UAG. Translation termination occurs when a stop codon enters the A site of the ribosome and is controlled essentially by the release factor eRF1, whose function is modulated by the GTPase eRF3  
15 (Stansfield, 1995; Zhouravleva, 1995). Translation termination is normally a highly efficient process. However, the misincorporation of an amino acid at the stop codon, also termed suppression or translational readthrough, can be influenced by several parameters, among which the local sequence context surrounding the stop codon seems to play a major role. The importance of the nucleotide immediately downstream the nonsense codon has been assessed in  
20 *in vitro* translational assays and has confirmed that the actual translational termination efficiency is strongly dependent on a tetranucleotide sequence (Manuvakhova et al., 2000).

The antibiotics belonging to the group of aminoglycosides have long been known to interfere with the decoding center of the ribosomal RNA (rRNA). These antibiotics cause misreading of the RNA code and can allow the insertion of alternative amino acids at the site of a  
25 stop codon (Palmer et al., 1979). Depending upon the dose, these drugs may inhibit protein synthesis. These observations have raised the possibility that diseases caused by nonsense mutations could be treated by aminoglycoside antibiotics. Some researchers have used this property of aminoglycosides in cell cultures or transgenic animals exhibiting nonsense codons within a structural gene to allow the translational machinery to translate the full mRNA, and thus  
30 complement the mutation. Using cultured mammalian cells, Burke and Mogg (1985) showed that the aminoglycoside antibiotics paromomycin and G-418 could partially restore the synthesis of a

full-size protein from a mutant gene with a premature UAG mutation. Later, G-418 and gentamicin were shown to restore the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) protein in a cell line carrying a nonsense mutation in CFTR (Bedwell et al., 1997; Howard et al., 1996). A similar study has been done in mutant mice exhibiting a premature stop codon in the dystrophin gene (Barton-Davis et al., 1999). These observations indicate that aminoglycosides are efficient both in cultured cells and in whole organisms to promote translational readthrough.

US 2002/0086427 A1 discloses an inducible eukaryotic expression system in which the expression of a desired gene can be activated or deactivated at the level of gene translation via an inducible signal. This is accomplished by introducing a mutation into the coding sequence of the gene of interest that causes a decrease or alteration of translation, e.g. a stop codon, and by contacting the eukaryotic cell containing the mutated gene of interest with an agent that suppresses the effect of the mutation, e.g. an aminoglycoside.

WO 03/014361 discloses a method for selection of single cell clones using stop codon-dependent translational coupling of marker gene expression with gene of interest expression, resulting in two recombinant gene products, a product encoded by the gene of interest and a fusion protein comprising the gene of interest combined with the selectable marker gene. The marker gene is e.g. a drug resistance gene or a reporter gene such as the GFP (green fluorescent protein) gene. The method may include use of a stop codon suppression mechanism, e.g. a SECIS element (selenocystein insertion sequence) to obtain insertion of the amino acid selenocystein at an UGA stop codon.

It has now been found that aminoglycoside antibiotics may be used to selectively obtain translational readthrough for e.g. alternative production of soluble and membrane-bound or otherwise tagged or marked forms of a recombinant protein from the same vector. This finding has important implications for providing improved and advantageous methods for selection and evaluation of cell clones or individual cells, and for evaluation of protein expression.

## SUMMARY OF THE INVENTION

In its broadest aspect, the invention relates to various methods for screening or selecting cells expressing a polypeptide of interest, as well as for producing a polypeptide of interest from a selected cell, where the cells comprise an expression cassette comprising a gene of interest and

a sequence encoding one or more of a cell membrane anchoring peptide, a reporter peptide and an epitope tag, and further at least one stop codon downstream of the sequence encoding the polypeptide of interest.

In one general aspect, the invention relates to methods for screening or selecting cells  
5 expressing a desired level of a polypeptide of interest, or for evaluating recombinant polypeptide expression in a population of cells, where the cells comprise an expression cassette comprising, in sequence, a coding sequence for a polypeptide of interest, a stop codon, and a coding sequence for at least one of a cell membrane anchoring peptide, a reporter peptide or an epitope tag.

A particular embodiment of this aspect of the invention relates to a method for screening  
10 or selecting cells expressing a desired level of a polypeptide of interest, comprising:

- a) providing a plurality of cells each comprising an expression cassette comprising a first nucleic acid sequence encoding the polypeptide of interest, at least one stop codon downstream of the first nucleic acid sequence, and a second nucleic acid sequence encoding a cell membrane anchoring peptide, a reporter peptide or an epitope tag downstream of the stop  
15 codon;
- b) cultivating the cells in the presence of a termination suppression agent under conditions that allow expression of the polypeptide of interest; and
- c) selecting at least one cell expressing the polypeptide of interest fused to a cell membrane anchoring peptide, a reporter protein or an epitope tag.

Another particular embodiment of this aspect of the invention relates to a method for  
20 evaluating recombinant protein expression in a population of cells, comprising:

- a) providing a plurality of cells each comprising an expression cassette comprising a first nucleic acid sequence encoding a recombinant polypeptide, at least one stop codon downstream of the first nucleic acid sequence, and a second nucleic acid sequence encoding a  
25 cell membrane anchoring peptide, a reporter peptide or an epitope tag downstream of the stop codon;
- b) cultivating the cells in the presence of a termination suppression agent under conditions that allow expression of a fusion protein comprising the recombinant polypeptide and the cell membrane anchoring peptide, reporter peptide or epitope tag; and
- c) sorting the cells to select at least one cell expressing the fusion protein at a desired  
30 level and/or with a desired uniformity.



A second general aspect of the invention relates to methods that allow alternate expression of different polypeptides from a single cell or cell line, for example i) a soluble, untagged polypeptide or ii) a membrane-bound or tagged polypeptide; or i) a membrane-bound, untagged polypeptide or ii) a membrane-bound, tagged polypeptide.

A particular embodiment of this aspect of the invention relates to a method for alternately expressing either i) a soluble, untagged polypeptide or ii) a membrane-bound or tagged polypeptide from a single cell or cell line, comprising:

a) providing a plurality of cells each comprising an expression cassette comprising a first nucleic acid sequence encoding the polypeptide of interest, at least one stop codon downstream of the first nucleic acid sequence, and a second nucleic acid sequence encoding a cell membrane anchoring peptide, a reporter peptide or an epitope tag downstream of the stop codon;

b) cultivating the cells in the presence of a termination suppression agent under conditions that allow expression of the polypeptide of interest;

c) selecting at least one cell expressing the polypeptide of interest fused to a cell membrane anchoring peptide, a reporter peptide or an epitope tag; and

d) cultivating said selected cell in the absence of a termination suppression agent to obtain expression of the polypeptide of interest as a soluble polypeptide.

A further embodiment of this aspect of the invention relates to a method for alternately expressing i) a membrane-bound, untagged polypeptide or ii) a membrane-bound tagged polypeptide from a single cell or cell line, comprising:

a) providing a plurality of cells each comprising an expression cassette comprising a first nucleic acid sequence encoding the polypeptide of interest and a cell membrane anchoring peptide, at least one stop codon downstream of the first nucleic acid sequence, and a second nucleic acid sequence encoding a reporter peptide or an epitope tag downstream of the stop codon;

b) cultivating the cells in the presence of a termination suppression agent under conditions that allow expression of the polypeptide of interest and the cell membrane anchoring peptide;

c) selecting at least one cell expressing a fusion protein comprising the polypeptide of interest, the cell membrane anchoring peptide, and a reporter peptide or an epitope tag; and

d) cultivating said selected cell in the absence of a termination suppression agent to obtain expression of a protein comprising the polypeptide of interest in membrane-bound form without the reporter peptide or epitope tag.

In a further general aspect, the invention provides methods suitable for use as alternatives to conventional antibiotic-based selection of cells transformed with a gene of interest, whereby the resulting selected cells may be used for production of a polypeptide of interest without undesired expression of an antibiotic resistance gene. In one embodiment of this aspect, the invention relates to a method for screening or selecting cells expressing a polypeptide of interest from a population of cells, comprising:

a) transfecting a population of cells with an expression cassette comprising, in sequence, a gene of interest, at least one stop codon, and a cell targeting peptide, wherein the expression cassette does not comprise an antibiotic resistance gene;

b) cultivating the transfected population of cells in the presence of a termination suppression agent; and

c) selecting at least one cell expressing the polypeptide of interest fused to a cell targeting peptide.

In another embodiment of this aspect of the invention, a method is provided in which antibiotic resistance is used for selection or screening purposes in the presence of an aminoglycoside antibiotic and a non-aminoglycoside antibiotic, but where the selected cells do not express the antibiotic resistance gene under normal production conditions in the absence of an aminoglycoside antibiotic. This embodiment relates to a method for screening or selecting cells expressing a polypeptide of interest from a population of cells, comprising:

a) transfecting a population of cells with an expression cassette comprising, in sequence, a gene of interest, at least one stop codon, and an antibiotic resistance gene, wherein the antibiotic resistance gene provides resistance to a non-aminoglycoside antibiotic;

b) cultivating the transfected population of cells in the presence of an aminoglycoside antibiotic and the non-aminoglycoside antibiotic; and

c) selecting at least one cell which is able to grow in the presence of the non-aminoglycoside antibiotic.

## DESCRIPTION OF THE DRAWINGS

Figure 1 shows the details of the vector *pLenti6-PC-GPI*.

Figure 2 shows the details of the vector *pLenti6-PC-UAAC-GPI*.

Figure 3 shows the details of the vector *pLenti6-PC-UGAC-GPI*.

Figure 4 shows the results of FACS analysis of cell surface expression of protein C (PC) and a GPI anchor with or without a stop codon and in the presence of different amounts of the aminoglycoside antibiotic Geneticin.

Figure 5 shows the results of FACS sorting and analyses of transgenic cell lines harboring the PC-UAAC-GPI construct, and that have been treated (B) or not treated (A) with Geneticin. Cells whose fluorescence was included in either the gate P2 or P3 of (B) were individually sorted and grown further prior to FACS analysis of membrane-anchored PC (C).

Figure 6 shows a comparison of protein C (PC) activity of 26 individual clones compared to the relative fluorescence of the respective clones determined by FACS.

Figure 7 shows the details of the vector *Retro-IFN-UGAC*.

Figure 8 shows the results of FACS analysis of three different clones for uniformity of recombinant protein expression within the cell populations.

Figure 9 shows the details of the vector *pCDNA6-FVII-UAA-EGFPd*.

Figure 10 shows the details of the vector *pCDNA6-ARI-UAA-V5*.

Figure 11 shows the details of the vector *pCDNA6-FVII-UAA-GPI*.

## DETAILED DISCLOSURE OF THE INVENTION

This invention provides, in one embodiment, a system that permits the efficient selection of cell lines expressing high levels of recombinant proteins by using Fluorescence-Activated Cell Sorting (FACS) and that relies on the property of aminoglycoside antibiotics to promote translational readthrough. The expression cassette is, for example, composed of a recombinant gene of interest (GOI) to be expressed into host cells, followed by a stop codon and a cell membrane anchoring signal. Any one of the three stop codons (UAA, UAG and UGA) in various tetranucleotide contexts can be chosen, depending on the background levels of suppression that are desired, as well as aminoglycoside-dependent inducibility and maximal readthrough levels upon aminoglycoside treatment. In the presence of aminoglycosides, translational readthrough is promoted and a subset of recombinant protein is produced as the recombinant protein fused to the cell membrane anchor signal. As a result, this fusion protein is displayed at the surface of host cells, and cells displaying high levels of membrane-anchored recombinant protein can be selected

by FACS. After cell sorting, cells are cultivated in the absence of aminoglycoside to allow efficient translational termination and production of high levels of soluble recombinant protein.

In another embodiment of the invention, the membrane anchoring signal can be replaced by a reporter gene such as the Green Fluorescent Protein (GFP) or an epitope tag such as the V5 epitope. In the presence of aminoglycosides, translational readthrough is promoted and as a result, a tagged version of the recombinant protein is produced. This allows the easy detection or quantification of recombinant protein expression by western blots or ELISA for example. If only production of native recombinant protein is desired, cells are grown in the absence of aminoglycosides to allow efficient translational termination. Furthermore, if the recombinant protein is a membrane-anchored protein, such as some hormone receptors, the aminoglycoside-mediated readthrough allows sorting of cell lines by FACS using detection antibodies targeted against the reporter gene or epitope. After cell sorting, the aminoglycoside antibiotic is removed from the culture medium to allow the production of untagged recombinant protein.

In another embodiment of the invention, both a reporter gene (or an epitope) and a membrane anchoring signal are translationally fused to the GOI that is followed by a stop codon. The resulting expression cassette (GOI-stop codon-reporter gene-membrane anchoring signal) typically allows efficient FACS-based selection of aminoglycoside-treated cells expressing high levels of recombinant protein because the fusion protein is targeted to the cell membrane. Additionally, the reporter protein or epitope tag, which is downstream of the termination signal, can be used as a target for specific antibodies during the FACS sorting. Alternatively, the reporter protein can be a protein exhibiting natural fluorescent properties (e.g. GFP). When soluble recombinant protein expression is desired, aminoglycosides are removed from the culture medium to allow efficient translational termination. As a result, the native recombinant protein alone may be produced from the same cell or vector used to produce the anchored or tagged version of the polypeptide of interest.

Unless otherwise defined herein or below in the remainder of the specification, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the present invention belongs.

A "nucleic acid sequence" or "polynucleotide sequence" is a nucleic acid (which is a polymer of nucleotides (A,C,T,U,G, etc. or naturally occurring or artificial nucleotide analogues)) or a character string representing a nucleic acid, depending on context. Either the given nucleic

acid or the complementary nucleic acid can be determined from any specified polynucleotide sequence.

Similarly, an "amino acid sequence" is a polymer of amino acids (a protein, polypeptide, etc.) or a character string representing an amino acid polymer, depending on context. Either the  
5 given nucleic acid or the complementary nucleic acid can be determined from any specified polynucleotide sequence.

The terms "protein", "peptide" or "polypeptide" may be used interchangeably herein to refer to polymers of amino acids, without any of these terms being limited to an amino acid sequence of a particular length. The terms "protein of interest" or "polypeptide of interest" may  
10 similarly be used interchangeably in the present context. These terms are intended to include not only full-length proteins but also e.g. fragments or truncated versions, variants, domains, etc. of any given protein or polypeptide. Similarly, the term "peptide" as used herein includes full-length proteins as well as e.g. shorter peptides of any given length depending on the context.

The polypeptide of interest is not limited to any particular protein or group of proteins,  
15 but may on the contrary be any protein, of any function or origin, which one desires to select and/or express by the methods described herein. The polypeptide of interest may thus be a therapeutic protein such as a cytokine, an antibody, a hormone or a therapeutic enzyme. Alternatively, the polypeptide of interest may e.g. be an industrial enzyme.

The polypeptide of interest can be a mature protein or a precursor form thereof, or a  
20 functional fragment thereof that essentially has retained a biological activity of the mature protein.

The polypeptide can be a therapeutic polypeptide useful in human or veterinary therapy, i.e. a polypeptide that is physiologically active when introduced into the circulatory system of or otherwise administered to a human or an animal; a diagnostic polypeptide useful in diagnosis; or  
25 an industrial polypeptide useful for industrial purposes, such as in a manufacturing process where the polypeptide constitutes a functional ingredient or where the polypeptide is used for processing or other modification of raw ingredients during manufacturing.

The polypeptide can be of mammalian origin, e.g. of human, porcine, ovine, ursine, murine, rabbit, donkey, or bat origin, of microbial origin, e.g. of fungal, yeast or bacterial origin,  
30 or can be derived from other sources such as from venom, or from a leech, frog or mosquito. In

the case of a therapeutic polypeptide, this is preferably of human origin, while an industrial polypeptide of interest is often of microbial origin.

Specific examples of groups of polypeptides that may be selected or expressed according to the invention include: an antibody or antibody fragment, an immunoglobulin or  
 5 immunoglobulin fragment, a plasma protein, an erythrocyte or thrombocyte protein, a cytokine, a growth factor, a profibrinolytic protein, a binding protein, a protease inhibitor, an antigen, an enzyme, a ligand, a receptor, and a hormone. Of particular interest is a polypeptide that mediates its biological effect by binding to a cellular receptor, when administered to a patient. In the case of an antibody, this can be a polyclonal or monoclonal antibody, and can be of any origin  
 10 including human, rabbit and murine origin. Preferably, the antibody is a human or humanized monoclonal antibody. Immunoglobulins of interest include IgG, IgE, IgM, IgA, and IgD and fragments thereof, e.g. Fab fragments. Specific antibodies and fragments thereof are those reactive with any of the proteins mentioned immediately below.

In the case of non-antibody polypeptides, these can be i) a plasma protein, e.g. a factor  
 15 from the coagulation system, such as Factor VII, Factor VIII, Factor IX, Factor X, Factor XIII, thrombin, protein C, antithrombin III or heparin co-factor II, Tissue factor inhibitor (e.g. 1 or 2), endothelial cell surface protein C receptor, a factor from the fibrinolytic system such as pro-urokinase, urokinase, tissue plasminogen activator, plasminogen activator inhibitor 1 (PAI-1) or plasminogen activator inhibitor 2 (PAI-2), the Von Willebrand factor, or an  $\alpha$ -1-proteinase  
 20 inhibitor, ii) an erythrocyte or thrombocyte protein, e.g. haemoglobin, thrombospondin or platelet factor 4, iii) a cytokine, e.g. an interleukin such as IL-1 (e.g. IL-1 $\alpha$  or IL-1 $\beta$ ), IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, a cytokine-related polypeptide, such as IL-1Ra, an interferon such as interferon- $\alpha$ , interferon- $\beta$  or interferon- $\gamma$ , a colony-stimulating factor such as GM-CSF or G-CSF, stem cell  
 25 factor (SCF), a binding protein, a member of the tumor necrosis factor family (e.g. TNF- $\alpha$ , lymphotoxin- $\alpha$ , lymphotoxin- $\beta$ , FasL, CD40L, CD30L, CD27L, O $\times$ 40L, 4-1BBL, RANKL, TRAIL, TWEAK, LIGHT, TRANCE, APRIL, THANK or TALL-1), iv) a growth factor, e.g. platelet-derived growth factor (PDGF), transforming growth factor  $\alpha$  (TGF- $\alpha$ ), transforming growth factor  $\beta$  (TGF- $\beta$ ), epidermal growth factor (EGF), vascular endothelial growth factor  
 30 (VEGF), somatotropin (growth hormone), a somatomedin such as insulin-like growth factor I (IGF-I) or insulin-like growth factor II (IGF-II), erythropoietin (EPO), thrombopoietin (TPO) or

angiopoietin, v) a profibrinolytic protein, e.g. staphylokinase or streptokinase, vi) a protease inhibitor, e.g. aprotinin or CI-2A, vii) an enzyme, e.g. superoxide dismutase, catalase, uricase, bilirubin oxidase, trypsin, papain, asparaginase, arginase, arginine deiminase, adenosin deaminase, ribonuclease, alkaline phosphatase,  $\beta$ -glucuronidase, purine nucleoside phosphorylase  
 5 or batroxobin, viii) an opioid, e.g. endorphins, enkephalins or non-natural opioids, ix) a hormone or neuropeptide, e.g. insulin, calcitonin, glucagons, adrenocorticotrophic hormone (ACTH), somatostatin, gastrins, cholecystokinins, parathyroid hormone (PTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), gonadotropin-releasing hormone, chorionic gonadotropin, corticotropin-releasing factor, vasopressin, oxytocin, antidiuretic hormones, thyroid-stimulating  
 10 hormone, thyrotropin-releasing hormone, relaxin, glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), prolactin, neuropeptide Y, peptide YY, pancreatic polypeptide, leptin, orexin, CART (cocaine and amphetamine regulated transcript), a CART-related peptide, melanocortins (melanocyte-stimulating hormones), melanin-concentrating hormone, natriuretic peptides, adrenomedullin, endothelin, exendin, secretin, amylin (IAPP; islet amyloid polypeptide  
 15 precursor), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP), agouti and agouti-related peptides or somatotropin-releasing hormones, or x) another type of protein or peptide such as thymosin, bombesin, bombesin-like peptides, heparin-binding protein, soluble CD4, pigmentary hormones, hypothalamic releasing factor, malanotonins, phospholipase activating protein, a detoxifying enzyme such as acyloxyacyl hydrolase, or an  
 20 antimicrobial peptide.

In the case of an industrial polypeptide, this is typically an enzyme, in particular a microbial enzyme used in products or in the manufacture of products such as detergents, household articles, personal care products, agrochemicals, textile, food products, in particular bakery products, feed products, or in industrial processes such as hard surface cleaning. The  
 25 industrial polypeptide is normally not intended for internal administration to humans or animals. Specific examples include hydrolases, such as proteases, lipases or cutinases, oxidoreductases, such as laccase and peroxidase, transferases such as transglutaminases, isomerases, such as protein disulphide isomerase and glucose isomerase, cell wall degrading enzymes such as cellulases, xylanases, pectinases, mannanases, etc., amylolytic enzymes such as endoamylases,  
 30 e.g. alpha-amylases, or exo-amylases, e.g. beta-amylases or amyloglucosidases, etc.

The stop codon, also known as a chain termination codon, used in the method of the invention may be any one or more of three codons, UAA, UAG and UGA, that signal termination of synthesis of a protein. Although expression cassettes for use in methods of the invention will often comprise only a single stop codon upstream of the coding sequence for the cell membrane anchoring peptide, reporter peptide, epitope tag or antibiotic resistance gene, it is also possible to use a series of two or more stop codons, e.g. two, three, four or five stop codons, which may be the same or different. As will be described in more detail below, there is generally a very low level of stop codon readthrough even in the absence of a chain termination agent. Depending on factors such as the natural level of background readthrough for a given stop codon in a given construct and the aim of a particular selection method according to the invention, it may in some cases be desirable to use more than one stop codon in order to further reduce background readthrough. Similarly, readthrough levels with and without a termination suppression agent may also be adjusted by selection of a suitable stop codon when only a single stop codon is used.

In addition to the possible use of multiple stop codons following the gene of interest, it will often be advantageous to use multiple stop codons downstream of the sequence encoding the cell membrane anchoring peptide, reporter peptide, epitope tag or antibiotic resistance gene. The use of multiple stop codons in this position, e.g. up to about 10 stop codons, such as up to about 6 or 8 stop codons, such as about 2, 3, 4 or 5 stop codons, will ensure efficient termination of translation even in the presence of the termination suppression agent.

The term "cell membrane anchoring peptide" refers to a peptide or protein that serves to anchor the polypeptide of interest to a cell membrane, either directly or indirectly. Indirect anchoring refers to situations in which the cell membrane anchoring peptide is not anchored in the cell membrane itself, but rather is indirectly attached to the lipid membrane bilayer as in the case of the GPI (glycosyl-phosphatidylinositol) anchor. Direct anchoring refers to situations in which the cell membrane anchoring peptide is directly embedded in and anchored to the lipid bilayer of the membrane. Polypeptides which are anchored to the cell membrane via an anchoring peptide will be displayed at the surface of the cell and can thus be identified, e.g. by FACS, or alternatively by other methods such as other fluorescence-based methods, ELISA or other affinity-based methods, or radioactivity-based methods. A preferred method is FACS, however, due to its high-throughput screening capacity that allows rapid and efficient screening of very large cell populations.



For purposes of screening using e.g. FACS, the cell membrane targeting signal is normally positioned at the COOH end of the protein fusion (downstream of the stop codon except where otherwise indicated herein). Additionally, it is important that the soluble part of the protein (i.e. the polypeptide of interest) is displayed on the right side of the membrane (the extracellular side) for subsequent antibody/ligand interaction during FACS. A preferred anchoring peptide is the GPI anchor. An example of another anchoring domain suitable for use in the methods of the invention is the C-terminal transmembrane anchoring domain of platelet derived growth factor receptor (PDGFR).

In a preferred embodiment, fusion proteins comprising a polypeptide of interest fused to a cell membrane anchoring peptide are sorted using Fluorescence-Activated Cell Sorting (FACS). In the context of the present invention, FACS sorting of membrane-bound fusion proteins is particularly advantageous, since it allows rapid screening of large numbers of cells to identify those in which the termination suppression agent has resulted in translational readthrough, as only these cells will express the polypeptide of interest at the cell surface in the form of a fusion protein comprising the polypeptide of interest and the cell membrane anchoring peptide. Once these cells have been identified by FACS, they can then be cultured in the absence of the termination suppression agent to result in production of the polypeptide of interest as a soluble polypeptide without the anchoring peptide. Surprisingly, the inventor has found that there is a positive and statistically significant correlation between fluorescence, as determined by FACS, and soluble protein activity levels. Thus, FACS sorting can be used in the method of the invention not only for qualitative analysis to identify cells expressing a protein of interest, but can actually be used quantitatively to identify cells that express high levels of a given protein. It has further been found that the methods of the invention are advantageous for evaluating heterogeneity of protein expression, i.e. for identifying and selecting cells or cell clones that exhibit both a desired level and a desired uniformity of protein expression.

The term "reporter peptide" refers to a peptide or protein that may readily be assayed by suitable means, thereby allowing easy detection of fusion proteins comprising a polypeptide of interest and the reporter peptide. A number of different reporter peptides are well-known in the art and include green fluorescent protein, luciferase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase and chloramphenicol acetyltransferase (CAT).

An "epitope tag" refers to a short amino acid sequence that serves as an antibody recognition site (epitope), allowing detection of a fusion protein comprising the polypeptide of interest and the epitope tag e.g. by means of fluorescently labeled antibodies that bind to the tag. Numerous epitope tags are known in the art, and products for detecting epitope tags, e.g. antibodies such as fluorescently labeled antibodies, are commercially available. Examples of epitope tags include V5 (GKPIPNPLLGLDST), His<sub>6</sub> (HHHHHH), FLAG<sup>TM</sup> (DYKDDDDKG), HA (YPYDVPDYA), c-Myc (EQKLISEEDL), VSV-G (YTDIEMNRLGK), and HSV (QPELAPEDPED).

The expression cassette may if desired include sequences that code for two or more of a cell membrane anchoring peptide, a reporter peptide and an epitope tag. For example, it may comprise a cell membrane anchoring peptide together with either a reporter peptide or an epitope tag, thus allowing the polypeptide of interest to be displayed at the cell surface in the form of a membrane-anchored fusion protein which may be screened or selected not only by FACS but also via the reporter peptide or epitope tag. In this case, the stop codon will be located downstream of the coding sequence for the polypeptide of interest but upstream of the coding sequences for the anchoring peptide and the reporter peptide or epitope tag.

Alternatively, in particular for proteins which in their native form are targeted to the plasma membrane, e.g. hormone receptors, the stop codon may be located downstream of the sequence encoding the cell membrane anchoring peptide but upstream of the sequence encoding the reporter peptide. In this case, expression in the presence of an aminoglycoside results in a non-native fusion protein that can be sorted or selected e.g. by FACS or affinity chromatography on the basis of the reporter peptide, while expression in the absence of an aminoglycoside results in a "native-type" membrane-bound protein comprising the polypeptide of interest. The term "native-type" in this context refers to the fact that the fusion protein comprises a non-tagged form of the polypeptide of interest (where the polypeptide of interest may be a mutagenized form of a "native" polypeptide) that is naturally targeted to the cell membrane.

In a further alternative embodiment, an epitope tag or reporter peptide, in particular an epitope tag, may be included before the stop codon, with an anchoring peptide after the stop codon, to generate the following construct: gene of interest-tag-STOP-anchor. In this case, the method is suitable for selecting cell lines producing high levels of soluble tagged protein by

FACS. The tag may e.g. be a His tag, a V5 epitope tag, or any of the other tags or reporter peptides listed above.

The "termination suppression agent" is a chemical agent which is able to suppress translational termination resulting from the presence of a stop codon. In particular, the  
5 termination suppression agent is an antibiotic belonging to the aminoglycoside group. As explained above, aminoglycoside antibiotics are known for their ability to allow insertion of alternative amino acids at the site of a stop codon, thereby resulting in "readthrough" of a stop codon that otherwise normally would result in chain termination. Aminoglycoside antibiotics include Geneticin (G-418), gentamicin (gentamycin), paromomycin, hygromycin, amikacin,  
10 kanamycin, neomycin, netilmicin, paromomycin, streptomycin and tobramycin.

It will be understood by persons skilled in the art that even in the absence of a termination suppression agent, there will generally be a small level of background stop codon readthrough. The degree of background readthrough varies somewhat depending on the particular stop codon, including the tetranucleotide context, and readthrough may also vary among different  
15 aminoglycoside antibiotics. Similarly, for a given stop codon and termination suppression agent, the degree of translational readthrough may be adjusted by varying the concentration of the termination suppression agent. These differences in background readthrough and in translational readthrough obtained with different stop codons and termination suppression agents may be used advantageously in the context of the present invention in order to select stop  
20 codons/tetranucleotides and aminoglycosides that provide the desired result. For example, the background readthrough of the UAA stop codon is lower than for the UGA stop codon, while higher translational readthrough rates are obtainable using, e.g., Geneticin with a UGA stop codon than with a UAA stop codon.

In one experiment with Geneticin, for example, the present inventor obtained up to about  
25 25% FACS-positive cells for the UGA stop codon (UGAC tetranucleotide), but only up to about 10% FACS-positive cells for the UAA stop codon (UAAC tetranucleotide). The background levels of FACS-positive cells in the absence of Geneticin in this case were about 13% and 0.5%, respectively, for UGAC and UAAC. By way of example, the UAA stop strategy may therefore be used for selecting high-expressing clones by FACS prior to production of soluble protein in the  
30 absence of an aminoglycoside, since the UAA construct has almost no background readthrough. Conversely, the UGA stop strategy may be a good alternative when maximum levels of

readthrough are wanted and background readthrough is not a concern, e.g. for functional library screening.

In the context of the present invention the term "soluble protein" or "soluble polypeptide" refers to the polypeptide of interest when expressed in soluble form without being fused to a cell membrane anchoring peptide. The soluble protein is thus generally obtained by expression in the absence of a termination suppression agent, whereby the at least one stop codon downstream of the first nucleic acid sequence effectively results in chain termination so that the polypeptide of interest is not membrane-bound. If desired, however, the soluble polypeptide may be expressed together with a reporter peptide or epitope tag, the coding sequence for the reporter peptide or tag in this case being located upstream of the stop codon(s).

As indicated above, one aspect of the invention provides methods suitable for use as alternatives to conventional antibiotic-based selection of cells transformed with a gene of interest. This allows for efficient selection of cells that have been transformed with the gene of interest, but has the advantage compared to antibiotic resistance-based selection methods of also allowing the resulting selected cells to be used for production of the polypeptide of interest without undesired expression of an antibiotic resistance gene. In a preferred embodiment of this aspect of the invention, no antibiotic resistance gene is present in the expression cassette comprising the gene of interest, the stop codon and the cell targeting peptide, i.e. in this case selection of cells expressing the polypeptide of interest is not based on antibiotic resistance. Instead, selection is related to the presence of the cell targeting peptide.

As used herein, a "cell targeting peptide" is a peptide or protein that targets the polypeptide of interest to the cell in which it is produced, i.e. to either the interior of the cell or linked to the exterior of the cell. Examples of suitable cell targeting peptides include membrane targeting peptides such as the GPI anchor, e.g. for cases where antibodies directed against the polypeptide of interest-cell targeting peptide fusion are to be used during FACS sorting, as well as any peptide that targets the fusion to cell compartments in the interior of the cell. Cell targeting peptides that may be used for intracellular targeting include e.g. a nuclear localization signal (NLS), a signal targeting the polypeptide to other sub-cellular compartments (e.g. the cytoplasm, mitochondria or endoplasmic reticulum), and cellular structures such as microtubules. For intracellular targeting, it will be understood that at least one of the proteins belonging to the

polypeptide of interest-cell targeting peptide fusion has intrinsic biochemical properties allowing its detection within the cell, for example by fluorescence.

In another embodiment of this aspect of the invention, selection of cells expressing the polypeptide of interest may be performed using a conventional antibiotic resistance technique, but where the presence of one or more stop codons downstream of the gene of interest and upstream of the antibiotic resistance gene ensures that the antibiotic resistance gene is not expressed under normal production conditions in the absence of an aminoglycoside antibiotic. Selection using this embodiment of the invention will normally employ two different antibiotics in the selection medium, i.e. an aminoglycoside antibiotic that results in translational readthrough and expression of the antibiotic resistance gene, and a non-aminoglycoside antibiotic used for the actual selection. Cells transformed with the expression cassette containing the gene of interest will thus express the antibiotic resistance gene, which provides resistance to the non-aminoglycoside antibiotic, but only in the presence of an aminoglycoside antibiotic that allows translational readthrough of the stop codon(s). Any non-aminoglycoside antibiotic may be used as the antibiotic for selection in this embodiment of the invention, e.g. ampicillin, bleomycin, phleomycin, spectinomycin, blasticidin, puromycin, zeocin, etc.

The present invention is applicable to any type of host cell from organisms in which translational stop codon readthrough is promoted in the presence of aminoglycosides, in particular eukaryotic cells such as mammalian cells or other animal cells, filamentous fungal cells, yeast cells, insect cells, and transgenic plants and animals. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)).

Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae*, *A. niger*, or *A. nidulans*, *Fusarium* and *Trichoderma*. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae*, *Schizosaccharomyces*, *Kluyveromyces*, *Pichia*, such as *P. pastoris* or *P. methanolica*, *Hansenula*, such as *H. polymorpha*, and *Yarrowia*. Examples of suitable insect host cells include a *Lepidoptera* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusia ni* cells (High Five) (US 5,077,214).

Preferably, the cells used in the methods of the invention are selected from mammalian cells and yeast cells.

Persons skilled in the art will be capable of selecting suitable vectors, expression control sequences and hosts for performing the methods of the invention. For example, in selecting a  
5 vector, the host must be considered because the vector must be able to replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the  
10 sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, possible toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and the ease of purification of the products  
15 coded for by the nucleotide sequence.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for the expression of polypeptides according to the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader sequence, polyadenylation  
20 sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences will include a promoter.

Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late  
25 promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) promoter, the Drosophila minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, J Mol Biol  
30 1987;196(4):947-50).

In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

5        Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence.

10       Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast  $\alpha$ -mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter and the inducible GAL promoter.

15       Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger*  $\alpha$ -amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator.

20       A signal peptide is present when the polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, when present, should be one recognized by the cell chosen for expression of the polypeptide. The signal peptide may be homologous (e.g. be that normally associated with the polypeptide in question) or heterologous (i.e. originating from another source) to the polypeptide or may be homologous or heterologous to the host cell, i.e. be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell.

25       In production methods of the present invention, cells are cultivated in a nutrient medium suitable for production of the polypeptide in question using methods known in the art. For example, the cells may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient  
30       medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to

published compositions (e.g., in catalogues of the American Type Culture Collection). When the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium.

As explained elsewhere herein, selection or screening of polypeptides according to the methods of the invention may be performed by any suitable means, e.g. by FACS in the case of membrane bound polypeptides or by suitable detection of a reporter peptide or epitope tag.

Polypeptides produced in accordance with the invention may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, ultra-filtration, extraction or precipitation. Purification may be performed by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation) or extraction (see, e.g., Protein Purification (2nd Edition), Janson and Ryden, editors, Wiley, New York, 1998).

The present invention also provides kits including the expression cassettes, expression vectors, cells and methods of the invention. Kits of the invention optionally comprise at least one of the following of the invention: (1) at least one kit component comprising an expression cassette as described herein suitable for performing a method of the invention; a cell or expression cassette comprising such an expression cassette; an aminoglycoside antibiotic; or a composition comprising at least one such component; (2) instructions for practicing any method described herein, instructions for using any component identified in (1) or any composition of any such component; (3) a container for holding said at least one such component or composition, and (4) packaging materials. Typically, the kit will comprise at least one component of (1) together with instructions for use and a container and/or packaging materials. The individual components of the kit may be packaged together or separately.

In a further aspect, the present invention provides for the use of any apparatus, component, composition, or kit described above and herein, for the practice of any method or assay described herein, and/or for the use of any apparatus, component, composition, or kit to practice any assay or method described herein.

The invention is further illustrated by the following non-limiting examples.



## EXAMPLES

### Example 1: Construction of pLenti6-PC-UAAC-GPI and pLenti6-PC-UGAC-GPI

For constructing the plenti6-UAAC-GPI vector, the coding sequence of human Protein  
 5 C (PC) was amplified by PCR (polymerase chain reaction) using the primers TBO077 (5'-  
 CCGTGACCAGTGCTTGGTCTTGC-3') and TBO103 (5'-  
 CAGTACGTGGGTTCCAGTTAAGGTGCCCAGCTCTTCTGGGGGGCTTCC-3'). The GPI  
 anchoring sequence was amplified by PCR using the primers TBO102 (5'-  
 CCAGAAGAGCTGGGCACCTTAACTGGAACCCACGTACTGCGACCTCGC-3') and  
 10 TBO104 (5'- ATCAGCGGTTTAACTTTCACTATTACTAGGGAGCGGTAGCGGTTTCC-  
 3'). The two resulting PCR products served as templates in a fusion PCR procedure using the  
 primers TBO077 and TBO104. The resulting PCR fragment was cleaved using the restriction  
 endonucleases PstI and PmeI and ligated into the vector *pLenti6-PC-GPI* (Figure 1) at the  
 corresponding endonuclease sites, giving *pLenti6-PC-UAAC-GPI* (Figure 2). This expression  
 15 vector harbors the PC sequence translationally fused to the GPI anchor sequence and a UAA stop  
 codon between the coding sequences of the PC and the GPI anchor. Additionally, four stop  
 codons follow the GPI anchor to efficiently terminate the translation even in the presence of  
 aminoglycosides.

For constructing the *plenti6-UGAC-GPI* vector, the coding sequence of the human PC  
 20 was amplified by PCR using the primers TBO077 (5'-CGGTGACCAGTGCTTGGTCTTGC-3')  
 and TBO108 (5'- CAGTACGTGGGTTCCAGTCAAGGT-  
 GCCCAGCTCTTCTGGGGGGCTTCC-3'). The GPI anchoring sequence was amplified by PCR  
 using the primers TBO107 (5'- CCAGAAGAGCTGGGCACCTTGACTGGAA-  
 CCCACGTACTGCGACCTCGC-3') and TBO104 (5'- ATCAGCGGTTTAACTTTCA-  
 25 CTATTACTAGGGAGCGGTAGCGGTTTCC-3'). The two resulting PCR products served as  
 templates in a fusion PCR procedure using the primers TBO077 and TBO104. The resulting PCR  
 fragment was cleaved using the restriction endonucleases PstI and PmeI and ligated into the  
 vector *pLenti6-PC-GPI* (Figure 1) at the corresponding endonuclease sites, giving *pLenti6-PC-  
 UGAC-GPI* (Figure 3). This expression vector harbors the protein C sequence translationally  
 30 fused to the GPI anchor sequence and a UGA stop codon between the coding sequences of the PC

and the GPI anchor. Additionally, four stop codons follow the GPI anchor to efficiently terminate the translation even in the presence of aminoglycosides.

### Example 2: Aminoglycoside-induced *in vivo* suppression of termination

5 To demonstrate that a recombinant gene expression vector as disclosed herein can be used for aminoglycoside-induced *in vivo* suppression of termination, the retroviral vectors *pLenti6-PC-UAAC-GPI* and *pLenti6-PC-UGAC-GPI* were used to transfect HEK293FT cells (Invitrogen) using the Lipofectamine™ 2000 (Invitrogen) transfection reagent. As a control, the retroviral vector *pLenti6-PC-GPI* was used to transfect HEK293FT cells and produce retrovirus  
10 particles. After 48 hours, supernatants containing retroviral particles were harvested, filter-sterilized to remove cell debris, and subsequently used to infect CHO-K1 cells. CHO-K1 cells were selected for resistance to the Blasticidin antibiotic at the concentration of 5 mg/l for 10 days. The resulting pools of Blasticidin-resistant cells were transferred into 6 culture flasks for each cell pool and grown to 25% confluency. To induce translational readthrough, the antibiotic  
15 Geneticin was added to the culture flasks at final concentrations ranging from 12.5 mg/l to 100 mg/l and flasks were incubated for another 48 hours at 37°C. Cells were detached from the flasks by trypsinization and were incubated with mouse anti-human PC monoclonal antibodies. Cells were subsequently washed and incubated with a secondary antibody (rabbit anti-mouse IgG, Phycoerythrin-labeled (DAKO R0439)). Labeled retroviral cell lines were analyzed by FACS for  
20 membrane-anchored recombinant PC using a FACScalibur™ (Becton Dickinson) instrument with an excitation wave length of 488 nm and an emission filter of 585 nm.

The results shown in Figure 4 indicate that both cell lines expressing the PC-UAAC-GPI and PC-UGAC-GPI reporters display PC at the cell surface in the presence of aminoglycoside. Moreover, the amount of recombinant protein that is detected (Y axis) is proportional to the  
25 Geneticin concentration. Furthermore, the amount of recombinant membrane-anchored PC is more abundant for the PC-UGAC-GPI construct than for the PC-UAAC-GPI construct, and this applies for all aminoglycoside concentrations that were assessed. This result is in accordance with aminoglycoside-mediated translational readthrough performed in an *in vitro* model (Manuvakhova et al., 2000). In contrast to the retroviral cell lines expressing the PC-UAAC-GPI  
30 and PC-UGAC-GPI reporters, the retroviral cell line expressing the PC-GPI reporter does not exhibit an increased amount of displayed recombinant protein in the presence of aminoglycoside.

This result was expected when considering that the expression cassette does not harbor a stop codon between the PC and GPI sequences. This result also confirms that both the UAAC and the UGAC tetranucleotides can be successfully used to modulate aminoglycoside-mediated *in vivo* translational readthrough.

5

### **Example 3. Efficient selection of cell clones expressing high levels of recombinant protein by FACS**

Until now, selection of clones expressing high levels of recombinant soluble protein has been a labor-intensive task that typically limits the number of clones that can be analyzed to a few hundred. Furthermore, because the expression of the selectable marker gene does not directly correlate with the expression levels of the gene of interest, most of the clones do not express satisfactory recombinant protein levels. FACS-based sorting of cells offers a high-throughput screening capacity that allows the daily analysis/sorting of cell populations greater than 1,000,000. However, no simple method is currently available for exploiting FACS approaches for isolating cells expressing soluble proteins based on the expression levels. A system that would allow the alternative production of membrane-anchored and soluble recombinant protein would therefore represent a valuable tool for the fast isolation of cells expressing very high protein levels.

To demonstrate that a recombinant gene expression vector as disclosed herein can be used for the selection of cell clones producing very high recombinant protein levels, the retroviral vector *pLenti6-PC-UAAC-GPI* was used to transfect HEK293FT cells using the Lipofectamine™ 2000 (Invitrogen) transfection reagent. After 48 hours, supernatants containing retroviral particles were harvested, filter-sterilized to remove cell debris, and subsequently used to infect CHO-K1 cells. CHO-K1 cells were selected for resistance to the Blasticidin antibiotic at the concentration of 5 mg/l for 10 days. The resulting pools of Blasticidin-resistant cells were transferred into two culture flasks and grown to 25% confluency. To induce translational readthrough, the antibiotic Geneticin was added to one culture flask at the final concentration of 100 mg/l and flasks were incubated for another 48 hours at 37°C. Cells were detached from the flasks by trypsinization and were incubated with mouse anti-human PC monoclonal antibodies. Cells were subsequently washed and incubated with a secondary antibody (rabbit anti-mouse IgG, Phycoerythrin (PE)-labeled (DAKO R0439)). Labeled CHO-K1 cells were sorted based on their relative fluorescence

at 585 nm using a FACSVantage™ cell sorter (Becton Dickinson) and using an excitation wave length of 488 nm. The results of FACS sorting of cells cultured in the presence of Geneticin are shown in Figure 5B, while Figure 5A shows the results for cells cultured without Geneticin. Cells exhibiting high fluorescence levels (gate P2, Figure 5B) or moderate fluorescence levels (gate P3, Figure 5B) were individually sorted into 96-well cell culture plates containing 0.1 ml of culture medium without Geneticin, to allow the production of soluble recombinant PC. Cell culture plates were incubated at 37°C for 5 days, after which the presence of individual cell colonies in each culture well was assessed by microscopy. Plates were incubated at 37°C until cells reached confluency, after which cells were transferred to larger culture wells (12-well culture plates containing 1 ml of medium in each well). Cells were grown to 25% confluency, after which fresh medium containing Geneticin at a final concentration of 100 mg/l was added to each well to induce translational readthrough. Culture plates were incubated for another 3 days at 37°C. Supernatants were saved and stored at -80°C until soluble PC activity assays were performed. Cells were trypsinized and then labeled with primary and secondary antibodies as described above, and subsequently analyzed for fluorescence using a FACScalibur™ cell analyzer (Becton Dickinson) with an excitation wave length of 488 nm and an emission filter of 585 nm..

Cells whose fluorescence was included in gate P2 or P3 of Figure 5B were individually sorted and grown further, after which analysis of membrane-anchored PC was performed by FACS. The results are presented in Figure 5C, which shows the detection of membrane-anchored PC in CHO-K1 clones expressing the PC-GPI fusion. 48 clones that exhibited low PC levels and 48 clones that exhibited high PC levels during FACS sorting were assayed for membrane-anchored PC levels by means of FACS analysis. The results confirm the relative recombinant protein expression levels that were observed during FACS sorting. Indeed, most clones that were sorted as high PC expressers exhibited higher recombinant protein levels than clones that were sorted as low PC expressers. These results indicate that the FACS sorting step was successful, both in measuring membrane-anchored PC levels and in the individual cell sorting.

To assess whether there is a correlation between membrane-anchored (*i.e.* aminoglycoside-induced readthrough) and soluble (*i.e.* efficient translational termination after the PC sequence) PC levels, supernatants from 26 clones exhibiting various membrane-anchored PC levels were measured in an enzymatic-based PC assay. The results of this assay, presented in Figure 6A, confirm that there is a correlation between soluble and membrane-anchored

recombinant protein expression levels. Indeed, clones exhibiting high membrane-anchored PC levels (as assessed by FACS analysis) exhibit high soluble PC levels whereas clones exhibiting low membrane-anchored PC levels exhibit relatively low soluble PC levels. FACS fluorescence levels and soluble PC activity levels were plotted on a new graph to further confirm the correlation between membrane-anchored and soluble recombinant protein expression levels (Figure 6B). Statistical analysis using a Pearson correlation test and assuming a Gaussian distribution indicates that there is less than 0.01% chance (P value 0.0001) that the data are due to random distribution.

In conclusion, the data presented here show that there is a direct correlation between soluble and membrane-anchored PC levels. As a result, the present invention provides a high throughput (HTP) FACS-based method for the efficient selection of individual clones expressing high levels of soluble recombinant proteins.

#### **Example 4: Alternative production of soluble or membrane-anchored recombinant protein from the same cell**

Many downstream applications after FACS cell sorting require the production of soluble recombinant protein. However, FACS cell sorting usually relies on the production of membrane- or intracellular-targeted recombinant protein. As a result, it is labor-intensive to subclone recombinant DNA from FACS hits into an expression vector suitable for soluble recombinant protein expression. This is particularly relevant when subcloning expression libraries exhibiting high complexities. Indeed, every manipulation of an expression library (PCR, ligation, cloning, transfection of target cells, etc.) results in the loss of library complexity. Additionally, the whole subcloning process is time-consuming and expensive. It would therefore be ideal to be able to produce a recombinant protein either as a soluble or membrane-anchored form from the same vector.

To demonstrate that a recombinant gene expression vector as disclosed herein can be used for the alternative production of soluble or membrane-anchored expression libraries, a library consisting of shuffled genes encoding mammalian interferon alpha (IFN $\alpha$ ) may be cloned into the retroviral vector pRetro, which contains, in sequence, the E-tag, the S-tag, the UGA translation termination signal, the V5 epitope and the GPI anchoring signal (see Figure 7 for complete plasmid features details). The resulting library, named pRetro-IFN $\alpha$ -lib, is used to

transfect HEK293FT cells using the Lipofectamin 2000 (Invitrogen) transfection reagent. After 48 hours, supernatants containing retroviral particles are harvested, filter-sterilized to remove cell debris, and used to infect CHO-K1 cells. CHO-K1 cells stably transfected with the recombinant DNA are selected for resistance to the Blasticidin antibiotic at the concentration of 5 mg/l for 10 days. To induce translational readthrough, the antibiotic Geneticin is added to the culture flask at the final concentration of 100 mg/l and the flask is incubated for another 48 hours at 37°C. Cells are detached from the flasks by trypsinization and are subsequently incubated with a monoclonal FITC-conjugated anti-V5 antibody (Invitrogen 46-0308). For investigating library clones exhibiting an improved binding of membrane-anchored recombinant interferon alpha to the interferon 2 receptor (IFNAR2), cells are incubated with a soluble Interferon 2 receptor (sIFNAR2) exhibiting 8 histidines at the C terminus of the protein. Cells are incubated with a biotinylated antibody targeted against the histidine tag. Quantification of sIFNAR2 is performed after addition of PE-labeled streptavidin that binds to biotin with excitation/emission filter sets of 488 nm and 530 nm, respectively. Cells exhibiting high fluorescence levels in the channel corresponding to the PE detection are sorted by FACS. The fluorescence levels obtained for the FITC channel are used as a reference to quantify membrane-anchored recombinant protein expression levels.

Following FACS sorting, independent cells (*i.e* clones) are cultured in 96-well culture plates without Geneticin to allow efficient translational termination and therefore promote the production of a soluble IF $\alpha$ -S-tag library. Cells are grown to confluency, after which supernatants are assayed for RNAase activity, which is mediated by the presence of the S-tag from the soluble IF $\alpha$ -S-tag chimeras.

#### **Example 5: Evaluation of heterogeneity of recombinant protein expression in cell populations by FACS analysis**

The production of recombinant protein in mammalian cells for therapeutic use requires the isolation of clones producing stable recombinant protein levels throughout generations. Unfortunately, cells deriving from the same original clones often exhibit substantial variations in recombinant protein expression levels. This can result from various causes, such as genetic instability or DNA methylation. As a result, recombinant cell lines that exhibit such discrepancies are inappropriate and must be discarded, in spite of their recombinant protein expression levels.

To demonstrate that a recombinant gene expression vector as disclosed herein can be used for the evaluation of heterogeneity of recombinant protein expression in cell clones, the retroviral vector *pLenti6-PC-UAAC-GPI* was used to transfect HEK293FT cells and produce retrovirus as described in Example 2. CHO-K1 cells were selected for resistance to the

5 Blasticidin antibiotic at the concentration of 5 mg/l for 10 days. To induce translational readthrough, the antibiotic Geneticin was added to the culture flask at the final concentration of 100 mg/l and the flask was incubated for another 48 hours at 37°C. Cells were detached from the flasks by trypsinization and were subsequently proceeded for FACS sorting as described in Example 2. Individual cells were sorted based on their fluorescence levels in 96-well culture  
10 plates containing 0.1 ml of culture medium without Geneticin, to allow the production of soluble recombinant PC. Cell culture plates were incubated at 37°C for 5 days, after which the presence of individual cell colonies in each culture well was assessed by microscopy. Plates were incubated at 37°C until cells reached confluency and cells were subsequently transferred to larger culture wells (12-well culture plates containing 1 ml medium each). Cells were grown to 25%  
15 confluency, after which fresh medium containing Geneticin at a final concentration of 100 mg/l was added to each well to induce translational readthrough. Culture plates were incubated for another 3 days at 37°C. Cells were trypsinized, then labeled with primary and secondary antibodies as described in Example 2, and subsequently analyzed for fluorescence using a FACScalibur™ cell analyzer (Becton Dickinson).

20 The results shown in Figure 8 confirm that the present invention allows the analysis of clones for uniformity of recombinant protein expression within the cell population. Indeed, FACS analysis of cell clones expressing the PC-GPI fusion reveals that some clones express relatively uniform PC-GPI protein levels (Figure 8A) whereas other clones exhibit much more variable recombinant protein levels that typically result in broader fluorescence peaks (Figure 8B).

25 Although the latter may express similar overall recombinant protein levels, they are not suitable as producer cell lines because the overall recombinant expression levels usually drop throughout generations, because cells that have partially or totally lost the ability to produce recombinant protein generally grow faster than cells expressing high levels of recombinant protein.

30 Additionally, FACS-based cell cloning sometimes leads to mistakes that result in the presence of more than one cell in each cell culture well. The presence of multiple clones in the same well is generally assessed by microscopy but is labor-intensive and may lead to incorrect

evaluations. An example of the presence of at least two different cell clones is presented in Figure 8C. Indeed, two clear individual peaks corresponding to cell populations expressing either low or high PC-GPI fusion protein levels are visible. The presence of two different cell populations may have arisen from failure at the FACS step, leading to the sorting of two cells in the same well.

- 5 Alternatively, it is possible that the cell population expressing the lowest PC levels has arisen from cells that have lost the ability to express the recombinant protein. The potential causes of such loss of expression capability are multiple and may include chromosome rearrangements or DNA methylation. In any event, such cell populations have to be discarded.

Until now, these clones exhibiting discrepancies in recombinant protein expression  
10 levels were not distinguishable from clones exhibiting stable recombinant protein expression levels at early stages post-cloning. Usually, regular enzymatic measurement of recombinant protein levels for many cell culture generations is required to be able to identify and thus eliminate such unstable clones. This step is labor intensive and drastically reduces the number of clones that can be analyzed.

- 15 The present invention provides an inexpensive alternative method that can be performed at early stages to analyze the stability of recombinant protein expression levels. Additionally, the invention permits detection of the presence of multiple cell populations expressing different recombinant protein expression levels in putative cell clones.

20 **Example 6: Alternative production of tagged or native soluble recombinant protein from the same cell**

- Recombinant proteins that are expressed in eukaryotic cells are often translationally fused to epitope tags that are usually short peptides for which specific antibodies are available. Alternatively, larger peptides that exhibit interesting enzymatic or biochemical properties  
25 (reporter peptides) can be translationally fused to the protein of interest. Tagging of recombinant protein by translational fusion with epitope tags or larger peptides has multiple applications, including protein purification via affinity matrix (e.g. poly-Histidine tag, V5 epitope), subcellular localization (GFP variants), western blotting and immuno-precipitation (epitope tags).

- However, the presence of peptide tags may interfere with the properties of the protein of  
30 interest, inhibiting protein folding, secretion, or enzymatic activities. Additionally, the presence



of a tag may be toxic for the cell or be simply not desired in downstream applications. As a result, the presence of a peptide tag may be desired only transiently.

The present invention represents an ideal tool for the alternative production of recombinant proteins in their native or tagged forms from the same cells.

5 In the following example, the sequence of the human coagulation factor seven (FVII) is translationally fused to the sequence of the Enhanced Green Fluorescent Protein (EGFP) using a PCR approach similar to that described in Example 1. In order to avoid possible internal translation re-initiation, the first Methionine (Met) codon of the EGFP is removed and replaced by the UAA translation termination triplet. The resulting DNA fragment is cloned into the vector  
10 *pCDNA6/myc-His-A* (Invitrogen) to give the vector *pCDNA6-FVII-UAA-EGFPd*, which contains four termination stop codons downstream of the EGFP gene (Figure 9).

The vector *pCDNA6-FVII-UAA-EGFPd* is used to transfect CHO-K1 cells using the Lipofectamine™ 2000 (Invitrogen) transfection reagent. After 48 hours, cells are selected for resistance to the Blasticidin antibiotic at the concentration of 5 mg/l for 10 days. The resulting  
15 pools of Blasticidin-resistant cells are transferred into two culture flasks and grown to 25% confluency. To induce translational readthrough, the antibiotic Geneticin is added to one culture flask at the final concentration of 100 mg/l and both flasks are incubated for another 48 hours at 37°C. Supernatants are harvested and assayed for the presence of FVII and EGFP proteins by ELISA and fluorescence assays, respectively.

20 In the presence of Geneticin, translational readthrough will occur and the EGFP reporter will be detected. In contrast, no EGFP fluorescence is expected above background levels in supernatants of cells grown in the absence of Geneticin. To confirm this result, a western blot using anti-FVII antibodies may be performed. A 45 kDa band is expected in supernatants from both Geneticin-treated and untreated samples. This band corresponds to the native FVII protein.  
25 A second band that exhibits a higher molecular size (72 kDa) is expected to be present only in supernatants from the Geneticin-treated cells. This larger band corresponds to a protein fusion comprising the FVII and the EGFP proteins.

**Example 7: Alternative production of tagged or native membrane-anchored recombinant protein from the same cell**

Some recombinant proteins that are produced in cells are targeted to the plasma membrane. This is the case for many hormone receptors. Because these proteins are also anchored into the plasma membrane of the host cells, it is possible to enrich for cells expressing high recombinant protein levels using a FACS approach. However, this approach requires that specific antibodies to the receptor are available for the detection of the recombinant protein. Alternatively, chemicals or peptides that are known to interact specifically with the recombinant protein can be used. If none are available, the present invention represents an attractive alternative because epitope or peptide tags that are translationally fused to the recombinant protein can be expressed in aminoglycoside-treated cells.

To demonstrate that the invention described herein can be used for the alternative production of tagged or native membrane-anchored recombinant protein from the same cell, the vector *pCDNA6-AR1-UAA-V5* is constructed (Figure 10). This vector drives the expression of the Adiponectin receptor 1 (AdipoR1) that belongs to the 7M transmembrane receptor family (Yamauchi et al., 2003). The *pCDNA6-AR1-UAA-V5* contains a UAA stop codon immediately downstream of the AdipoR1 sequence, as well as a sequence coding for the V5 epitope.

CHO-K1 cell lines stably transfected with this vector are generated as described in Example 5. Following generation of cell lines, cells are divided into two flasks and grown to 25% confluency. To induce translational readthrough, the antibiotic Geneticin is added to one culture flask at the final concentration of 100 mg/l, then both flasks are incubated for 48 hours at 37°C. Cells are detached from the flasks by trypsinization and are subsequently incubated with FITC-labeled anti-V5 monoclonal antibodies (Invitrogen 46-0308).

Labeled CHO-K1 cells are sorted based on their relative fluorescence at 530 nm using a FACSVantage™ cell sorter (Becton Dickinson) with an excitation wave length of 488 nm. Cells exhibiting high or moderate fluorescence levels are individually sorted into 96-well cell culture plates containing 0.1 ml of culture medium without Geneticin, to allow the production of recombinant native AdipoR1. Cell culture plates are incubated at 37°C for 5 days, after which the presence of individual cell colonies in each culture well is assessed by microscopy. Plates are incubated at 37°C until cells reach confluency and cells are subsequently transferred to larger culture wells (12-well culture plates containing 1 ml medium each). Cells are grown to 25%

confluency, after which fresh medium containing Geneticin at a final concentration of 100 mg/l is added to each well to promote translational readthrough. Culture plates are incubated for another 3 days at 37°C. Cells are trypsinized, then labeled with anti-V5 antibody as described above, and subsequently analyzed for fluorescence using a FACScalibur™ cell analyzer (Becton Dickinson).

5        48 clones that exhibit low V5 levels and 48 clones that exhibit high V5 levels during FACS sorting are assayed for membrane-anchored V5 levels by means of FACS analysis. These results are expected to confirm the relative recombinant protein expression levels that are observed during FACS sorting. It is expected that most clones that are sorted as high V5 expressers will exhibit higher recombinant protein levels than clones that are sorted as low V5  
10    expressers.

As a result, the present invention provides a high throughput (HTP) FACS-based method for the efficient selection of individual clones expressing high levels of membrane-anchored recombinant proteins.

#### 15    **Example 8: Selection of recombinant cell lines devoid of antibiotic resistance**

To obtain cell lines producing a recombinant protein of interest, classical methods rely on the presence of an additional recombinant gene that is carried by the DNA vector used during the transfection and that confer resistance to an antibiotic. After transfection, cells are cultivated in the presence of antibiotic concentrations known to inhibit cell growth or kill wild-type cells.

20    As a result, only cells that express the recombinant protein conferring resistance to the given antibiotic are able to grow.

Although the presence of the resistance marker provides a valuable method for selecting cells expressing a recombinant protein of interest, many downstream applications do not require the presence, or the expression, of this selectable marker. For example, the promoter driving the  
25    resistance marker gene is often a very strong promoter of viral origin that is constitutively active. As a result, the recombinant RNA coding for the selection marker may compete with other RNAs for protein production and may reduce the yields of the recombinant protein of interest. Furthermore, the massive production of RNA coding for the selection marker may trigger post-transcriptional gene silencing, and therefore may lead to reduced yields of the recombinant  
30    protein of interest. Another advantage of a method enabling the selection of cell lines devoid of antibiotic resistance is that it would eliminate the potential for horizontal transfer of the antibiotic

resistance selection marker gene to wild-type species, which represents a possible biohazard risk for the environment.

To demonstrate that the invention described herein can be used for the selection of recombinant cell lines devoid of antibiotic resistance, PCR is performed using the  
5 oligonucleotides 5'cgatgtacgggccagatatacgcg3' and CBprof314 (5' CCTCAGAAGCCA-TAGAGCCCCACCGCA 3') using the vector *pCDNA6-FVII-UAA-GPI* as a template. The resulting PCR product comprises the CMV promoter, the b-globin intron, the FVII gene, the UAA stop codon, the GPI anchor signal and the b-globin poly-adenylation signal (Figure 11). The PCR product is purified and subsequently used to transfect CHO-K1 cells. Following  
10 transfection, cells are incubated for 2 days in the presence of Geneticin at 100 mg/L to promote translational readthrough. Because cells that express the recombinant FVII protein also express the FVII-GPI protein fusion arising from aminoglycoside-mediated translational readthrough, transgenic cells can be selected by means of FACS based on membrane-anchored FVII detection. Following FACS, the cell pool that has been enriched for recombinant protein expressers is  
15 grown for two days in the absence of antibiotic, after which translational readthrough is induced by treating the cells with Geneticin at 100 mg/L. Cells are subjected to several rounds of FACS-based enrichment until most cells are positive for membrane-anchored FVII.

Once a pool of cells stably expressing the recombinant protein has been obtained, it is possible to subject the cell pool to individual cell cloning by means of FACS or other methods.

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 30

**CLAIMS**

1. A method for screening or selecting cells expressing a desired level of a polypeptide of interest, comprising:

5 a) providing a plurality of cells each comprising an expression cassette comprising a first nucleic acid sequence encoding the polypeptide of interest, at least one stop codon downstream of the first nucleic acid sequence, and a second nucleic acid sequence encoding a cell membrane anchoring peptide, a reporter peptide or an epitope tag downstream of the stop codon;

10 b) cultivating the cells in the presence of a termination suppression agent under conditions that allow expression of the polypeptide of interest; and

c) selecting at least one cell expressing the polypeptide of interest fused to a cell membrane anchoring peptide, a reporter peptide or an epitope tag.

15 2. A method for evaluating recombinant polypeptide expression in a population of cells, comprising:

a) providing a plurality of cells each comprising an expression cassette comprising a first nucleic acid sequence encoding a recombinant polypeptide, at least one stop codon downstream of the first nucleic acid sequence, and a second nucleic acid sequence encoding a cell membrane anchoring peptide, a reporter peptide or an epitope tag downstream of the stop codon;

20 b) cultivating the cells in the presence of a termination suppression agent under conditions that allow expression of a fusion protein comprising the recombinant polypeptide and the cell membrane anchoring peptide, reporter peptide or epitope tag; and

25 c) sorting the cells to select at least one cell expressing the fusion protein at a desired level and/or with a desired uniformity.

3. The method of claim 1 or 2, wherein the termination suppression agent is an aminoglycoside antibiotic.

4. The method of any of the preceding claims, wherein the cells are screened or selected by FACS.

5. The method of any of the preceding claims, wherein the second nucleic acid sequence encodes a cell membrane anchoring peptide, and wherein the at least one selected cell expresses a fusion protein comprising the polypeptide of interest fused to a cell membrane anchoring peptide, the fusion protein being displayed at the surface of said cell.

6. The method of claim 5, wherein the cell membrane anchoring peptide is a GPI anchor.

7. The method of any of claims 1-4, wherein the second nucleic acid sequence encodes a reporter peptide or an epitope tag.

8. The method of claim 7, wherein the second nucleic acid sequence encodes a reporter peptide selected from the group consisting of green fluorescent protein (GFP), luciferase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase and chloramphenicol acetyltransferase (CAT).

9. The method of claim 7, wherein the second nucleic acid sequence encodes an epitope tag selected from the group consisting of V5, His, FLAG<sup>TM</sup>, HA, c-Myc, VSV-G, and HSV.

10. The method of any of claims 7-9, wherein the expression cassette further comprises a nucleic acid sequence encoding a cell membrane anchoring peptide.

11. The method of any of claims 3-10, wherein the aminoglycoside antibiotic is selected from the group consisting of geneticin (G-418), gentamicin (gentamycin), paromomycin, hygromycin, amikacin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin and tobramycin.

12. The method of any of the preceding claims, wherein the cell is a eukaryotic cell.

13. The method of claim 12, wherein the cell is selected from the group consisting of mammalian cells, filamentous fungal cells, yeast cells and insect cells.

14. The method of any of the preceding claims, further comprising:

5 d) cultivating at least one selected cell in the absence of a termination suppression agent to obtain expression of the polypeptide of interest as a soluble polypeptide.

15. A method for alternately expressing i) a soluble, untagged polypeptide or ii) a membrane-bound or tagged polypeptide from a single cell or cell line, comprising:

10 a) providing a plurality of cells each comprising an expression cassette comprising a first nucleic acid sequence encoding the polypeptide of interest, at least one stop codon downstream of the first nucleic acid sequence, and a second nucleic acid sequence encoding a cell membrane anchoring peptide, a reporter peptide or an epitope tag downstream of the stop codon;

15 b) cultivating the cells in the presence of a termination suppression agent under conditions that allow expression of the polypeptide of interest;

c) selecting at least one cell expressing the polypeptide of interest fused to a cell membrane anchoring peptide, a reporter peptide or an epitope tag; and

20 d) cultivating said selected cell in the absence of a termination suppression agent to obtain expression of the polypeptide of interest as a soluble polypeptide.

16. The method of claim 15, wherein the termination suppression agent is an aminoglycoside antibiotic.

25 17. The method of claim 15 or 16, wherein the cells are screened or selected by FACS.

18. The method of any of claims 15-17, wherein the second nucleic acid sequence encodes a cell membrane anchoring peptide, and wherein the at least one selected cell expresses a fusion protein comprising the polypeptide of interest fused to a cell membrane anchoring peptide, the  
30 fusion protein being displayed at the surface of said cell.



19. The method of claim 18, wherein the cell membrane anchoring peptide is a GPI anchor.

20. The method of any of claims 15-17, wherein the second nucleic acid sequence encodes a reporter peptide or an epitope tag.

5

21. The method of claim 20, wherein the second nucleic acid sequence encodes a reporter peptide selected from the group consisting of green fluorescent protein, luciferase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase and chloramphenicol acetyltransferase (CAT).

10 22. The method of claim 21, wherein the second nucleic acid sequence encodes an epitope tag selected from the group consisting of V5, His, FLAG<sup>™</sup>, HA, c-Myc, VSV-G, and HSV.

23. The method of any of claims 20-22, wherein the expression cassette further comprises a nucleic acid sequence encoding a cell membrane anchoring peptide.

15

24. The method of any of claims 16-23, wherein the aminoglycoside antibiotic is selected from the group consisting of geneticin (G-418), gentamicin (gentamycin), paromomycin, hygromycin, amikacin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin and tobramycin.

20

25. The method of any of claims 15-24, wherein the cell is a eukaryotic cell.

26. The method of claim 25, wherein the cell is selected from the group consisting of mammalian cells, filamentous fungal cells, yeast cells and insect cells.

25

27. A method for alternately expressing i) a membrane-bound, untagged polypeptide or ii) a membrane-bound, tagged polypeptide from a single cell or cell line, comprising:

30 a) providing a plurality of cells each comprising an expression cassette comprising a first nucleic acid sequence encoding the polypeptide of interest and a cell membrane anchoring peptide, at least one stop codon downstream of the first nucleic acid sequence, and a second

nucleic acid sequence encoding a reporter peptide or an epitope tag downstream of the stop codon;

b) cultivating the cells in the presence of a termination suppression agent under conditions that allow expression of the polypeptide of interest and the cell membrane anchoring peptide;

c) selecting at least one cell expressing a fusion protein comprising the polypeptide of interest, the cell membrane anchoring peptide, and a reporter peptide or an epitope tag; and

d) cultivating said selected cell in the absence of a termination suppression agent to obtain expression of a protein comprising the polypeptide of interest in membrane-bound form without the reporter peptide or epitope tag.

28. The method of claim 27, wherein the termination suppression agent is an aminoglycoside antibiotic.

29. The method of claim 27 or 28, wherein the cells are screened or selected by FACS.

30. The method of any of claims 27-29, wherein the cell membrane anchoring peptide is a GPI anchor.

31. The method of any of claims 28-30, wherein the aminoglycoside antibiotic is selected from the group consisting of geneticin (G-418), gentamicin (gentamycin), paromomycin, hygromycin, amikacin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin and tobramycin.

32. The method of any of claims 27-31, wherein the cell is a eukaryotic cell.

33. The method of claim 32, wherein the cell is selected from the group consisting of mammalian cells, filamentous fungal cells, yeast cells and insect cells.

34. The method of any of claims 27-33, wherein the second nucleic acid sequence encodes a reporter peptide selected from the group consisting of green fluorescent protein, luciferase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase and chloramphenicol acetyltransferase (CAT).

5 35. The method of any of claims 27-33, wherein the second nucleic acid sequence encodes an epitope tag selected from the group consisting of V5, His, FLAG<sup>TM</sup>, HA, c-Myc, VSV-G, and HSV.

36. A method for screening or selecting cells expressing a polypeptide of interest from a  
10 population of cells, comprising:

a) transfecting a population of cells with an expression cassette comprising, in sequence, a gene of interest, at least one stop codon, and a cell targeting peptide, wherein the expression cassette does not comprise an antibiotic resistance gene;

b) cultivating the transfected population of cells in the presence of a termination  
15 suppression agent; and

c) selecting at least one cell expressing the polypeptide of interest fused to a cell targeting peptide.

37. The method of claim 36, wherein the cell targeting peptide is selected from the group  
20 consisting of cell membrane anchoring peptides, nuclear localization signals, signals targeting the polypeptide of interest to a non-nuclear sub-cellular compartment, and cellular structures.

38. The method of claim 37, wherein the cell targeting peptide is selected from the group consisting a GPI anchor; a signal targeting the polypeptide of interest to the cytoplasm,  
25 mitochondria or endoplasmic reticulum; and microtubules.

39. The method of any of claims 36-38, wherein the termination suppression agent is an aminoglycoside antibiotic.

40. The method of claim 39, wherein the aminoglycoside antibiotic is selected from the group consisting of geneticin (G-418), gentamicin (gentamycin), paromomycin, hygromycin, amikacin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin and tobramycin.

5 41. The method of any of claims 36-40, wherein the cells are screened or selected by FACS.

42. The method of any of claims 36-41, further comprising:

d) cultivating at least one selected cell in the absence of a termination suppression agent to obtain expression of the polypeptide of interest without the cell targeting peptide.

10 43. A method for screening or selecting cells expressing a polypeptide of interest from a population of cells, comprising:

a) transfecting a population of cells with an expression cassette comprising, in sequence, a gene of interest, at least one stop codon, and an antibiotic resistance gene, wherein the antibiotic resistance gene provides resistance to a non-aminoglycoside antibiotic;

b) cultivating the transfected population of cells in the presence of an aminoglycoside antibiotic and the non-aminoglycoside antibiotic; and

c) selecting at least one cell which is able to grow in the presence of the non-aminoglycoside antibiotic.

20 44. The method of claim 43, wherein the aminoglycoside antibiotic is selected from the group consisting of geneticin (G-418), gentamicin (gentamycin), paromomycin, hygromycin, amikacin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin and tobramycin.

25 45. The method of claim 43 or 44, wherein the non-aminoglycoside antibiotic is selected from the group consisting of ampicillin, bleomycin, phleomycin, spectinomycin, blasticidin, puromycin and zeocin.

46. The method of any of claims 43-45, wherein the cells are screened or selected by FACS.

30 47. The method of any of claims 43-46, further comprising:

d) cultivating at least one selected cell in the absence of any antibiotic to obtain expression of the polypeptide of interest without expression of the antibiotic resistance gene.

48. A kit suitable for performing the method of any of the preceding claims.

5

49. The kit of claim 48, comprising one or more of: (1) at least one kit component comprising an expression cassette as defined in any of claims 1-47; a cell or expression vector comprising said expression cassette; an aminoglycoside antibiotic; or a composition comprising at least one such component; (2) instructions for practicing a method as defined in any of claims 1-47, instructions for using any component identified in (1) or any composition of any such component; (3) a container for holding said at least one such component or composition, and (4) packaging materials.

10

Figure 1

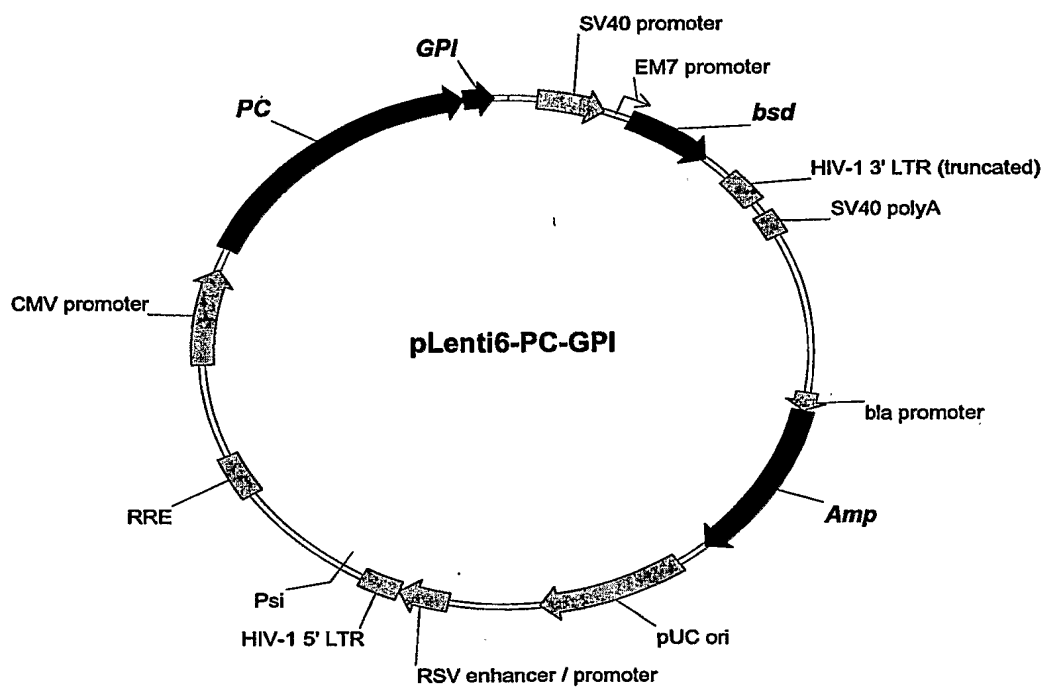


Figure 2

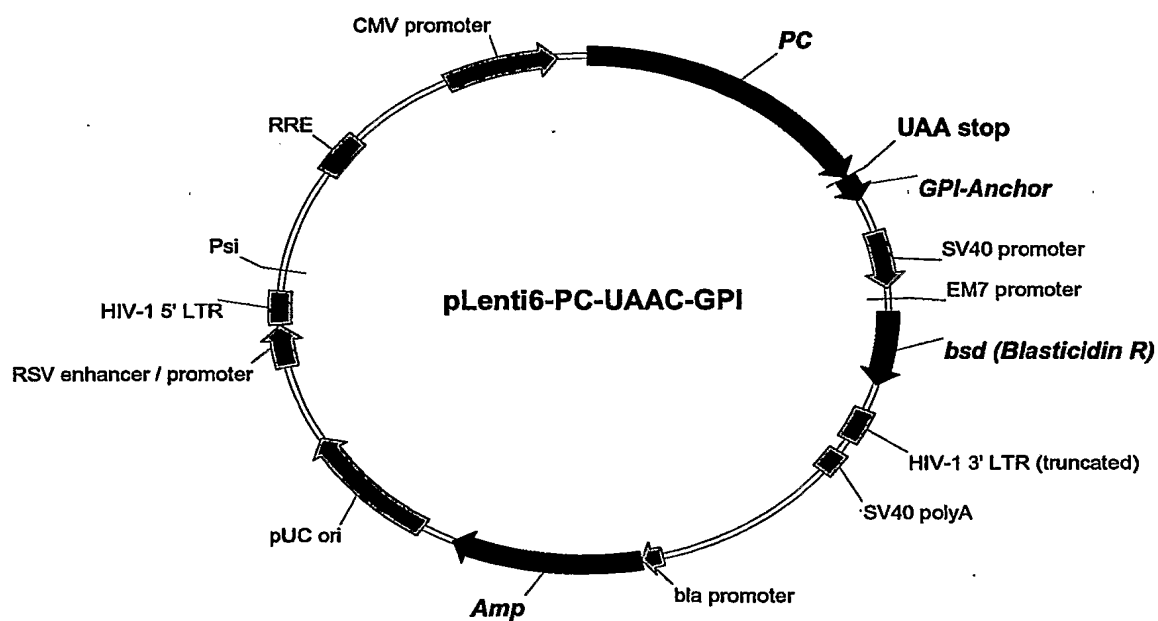


Figure 3

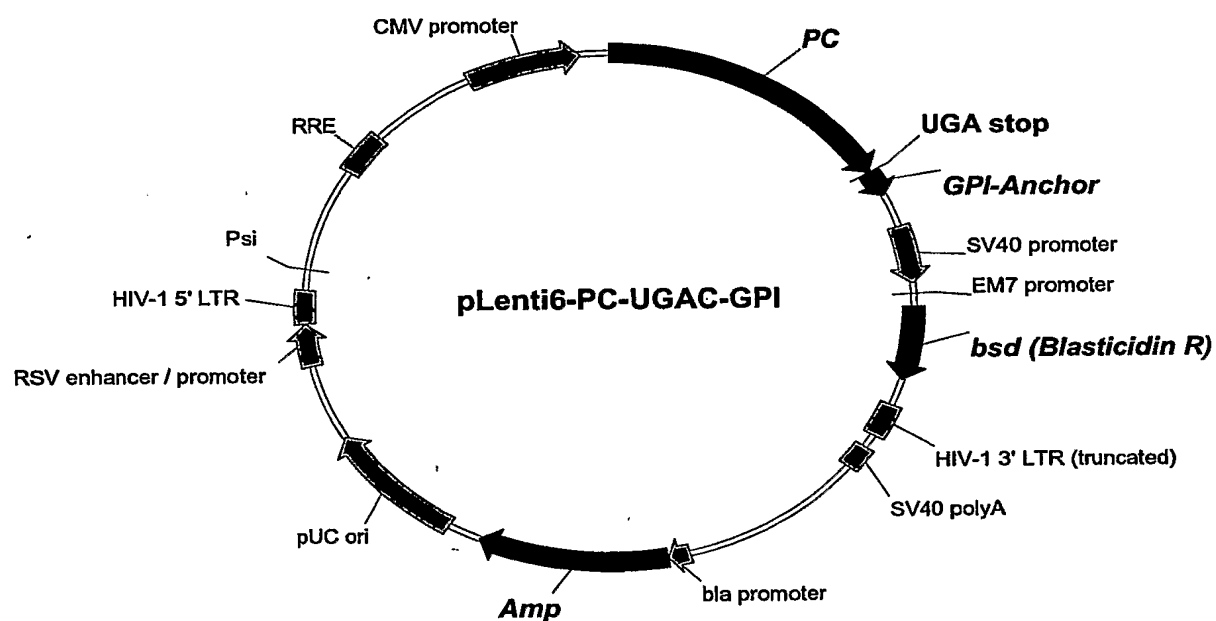




Figure 4

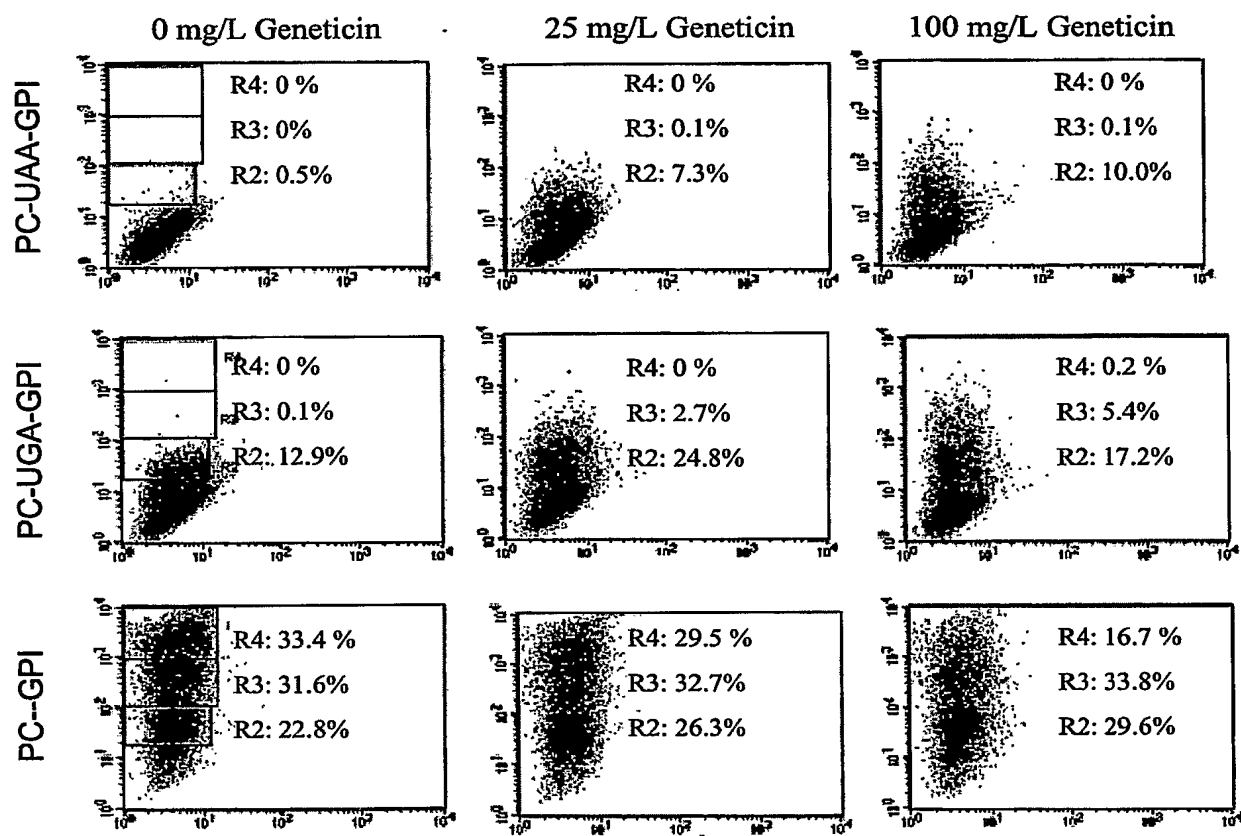
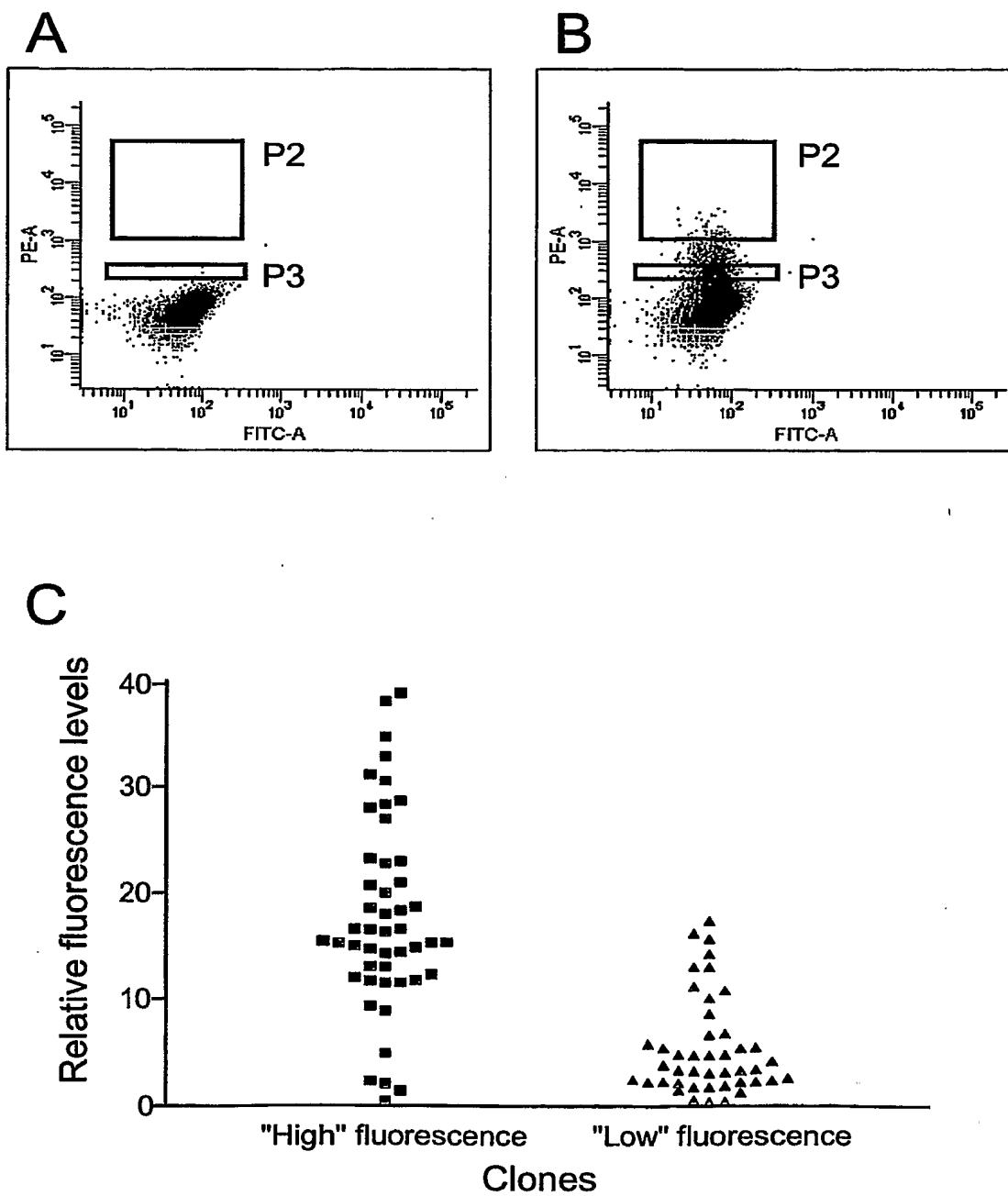
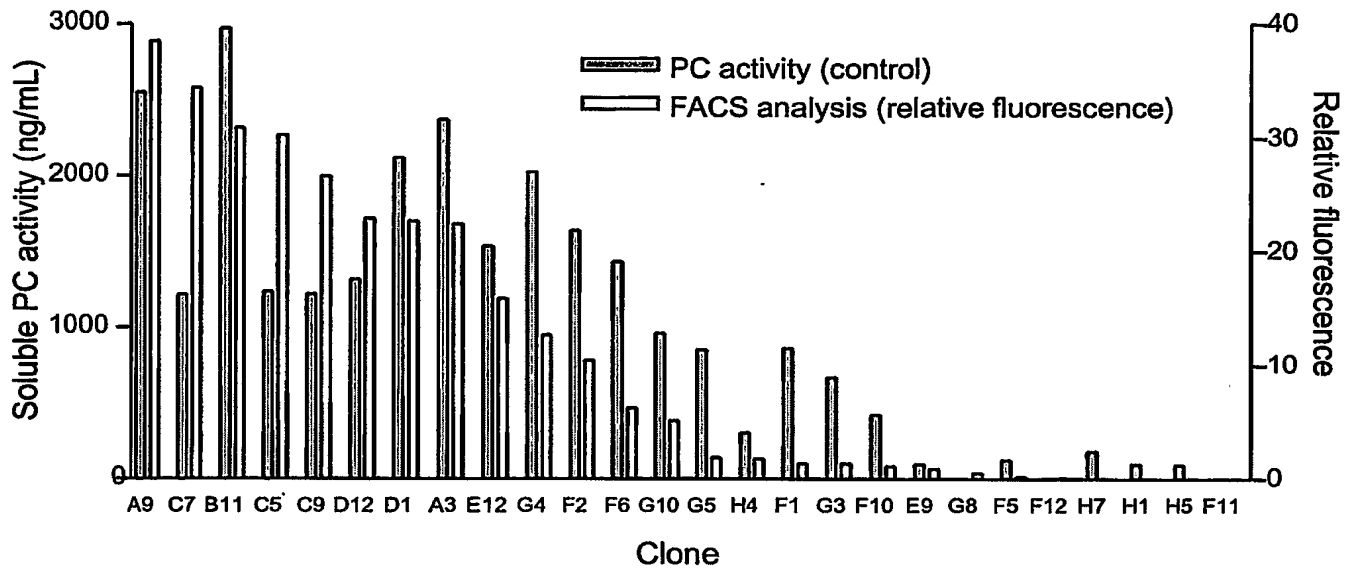


Figure 5



# Figure 6

## A



## B

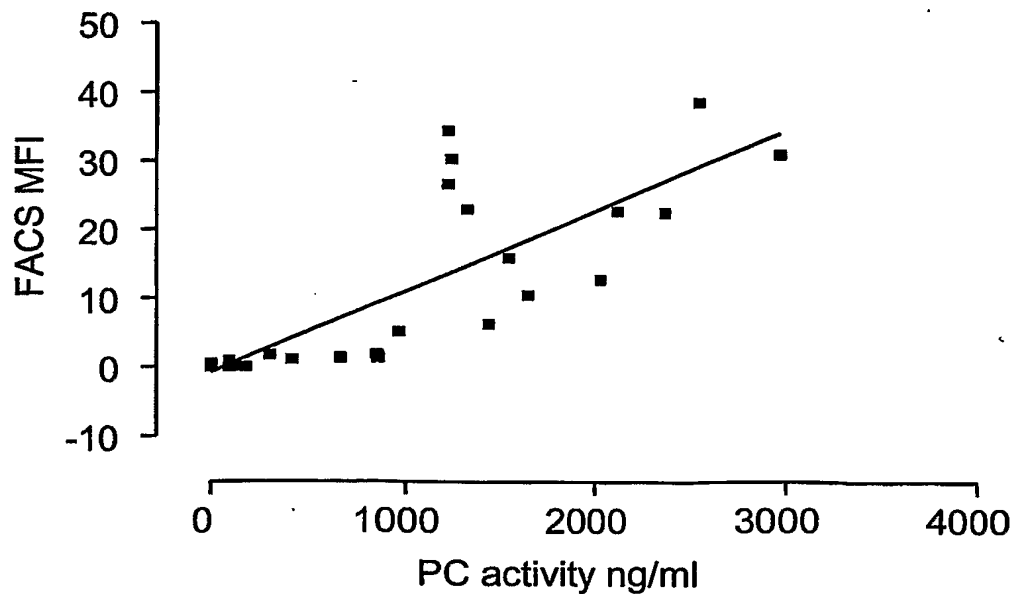
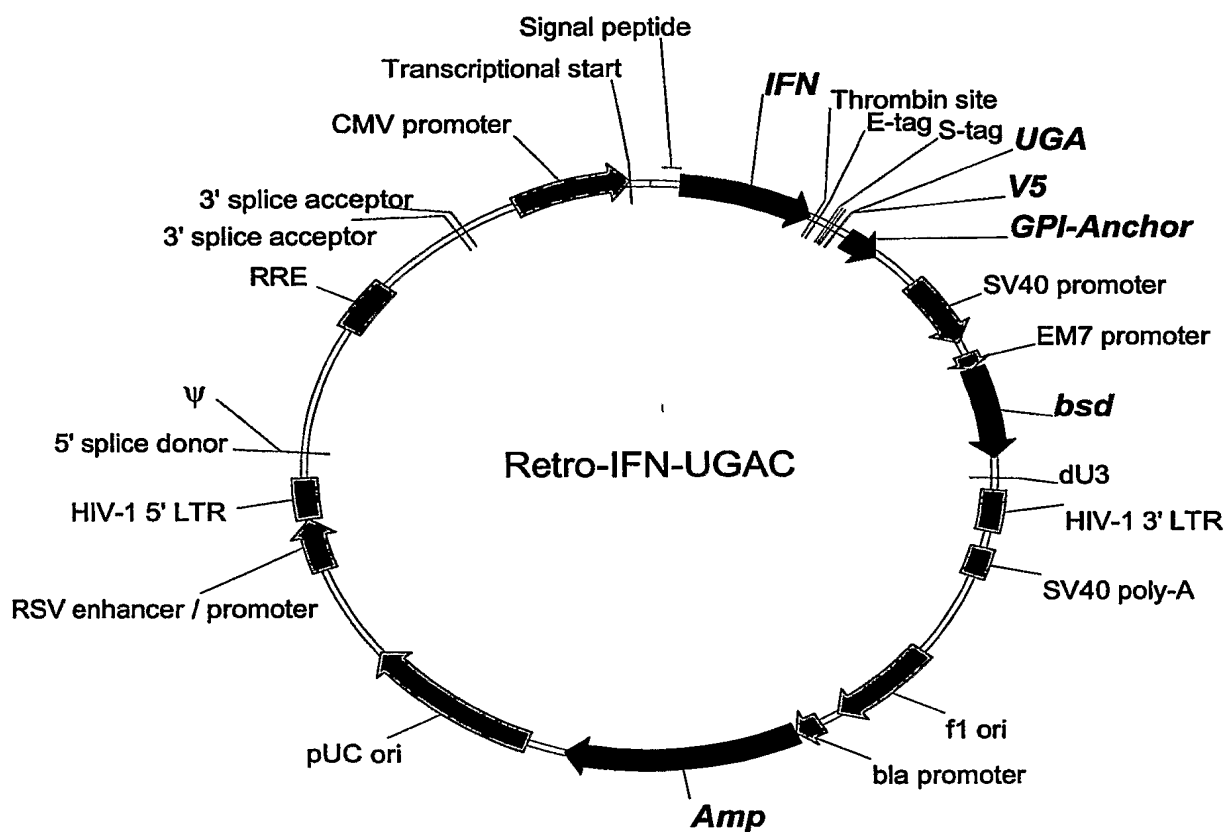
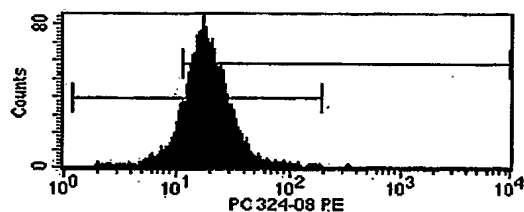


Figure 7



# Figure 8

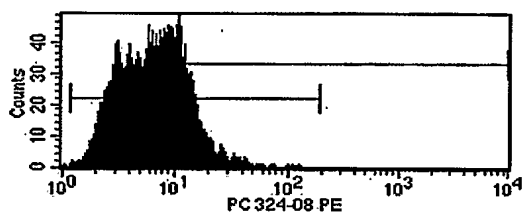
A



Histogram Statistics

Marker	Events	% Gated	Geo. Mean	CV
All	6751	100.00	18.20	57.62
% Pos. cells	5938	87.96	20.14	53.19
CV/Mean	6750	99.99	18.19	54.76

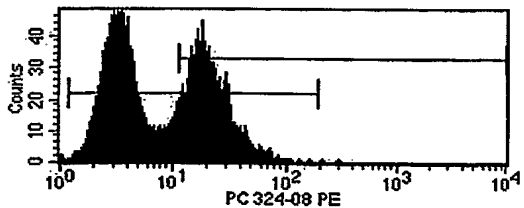
B



Histogram Statistics

Marker	Events	% Gated	Geo. Mean	CV
All	6821	100.00	6.31	75.12
% Pos. cells	1275	18.69	15.13	49.51
CV/Mean	6820	99.99	6.31	75.11

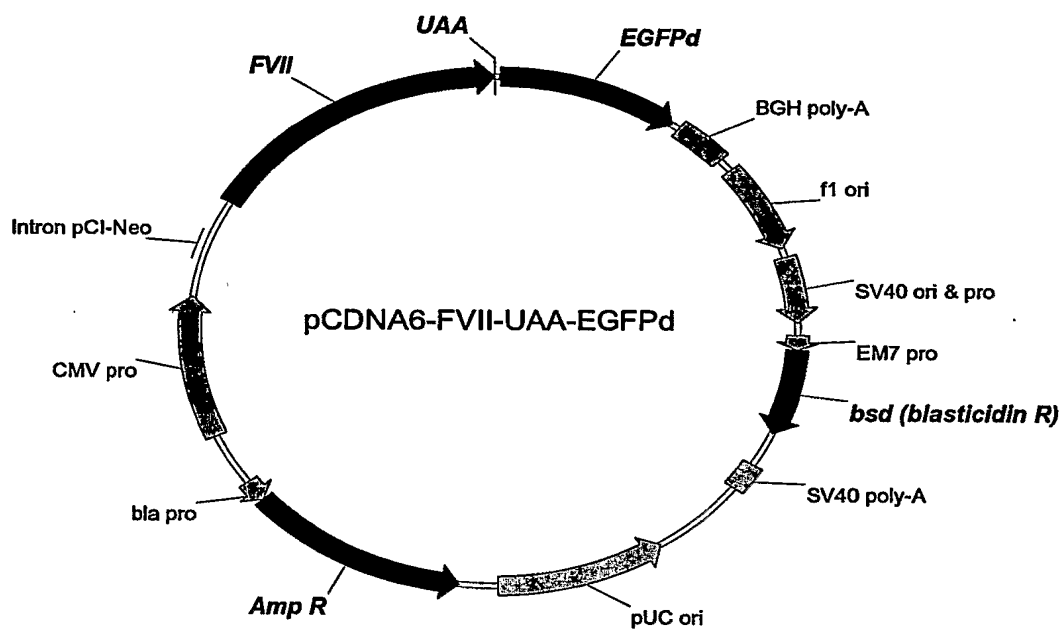
C



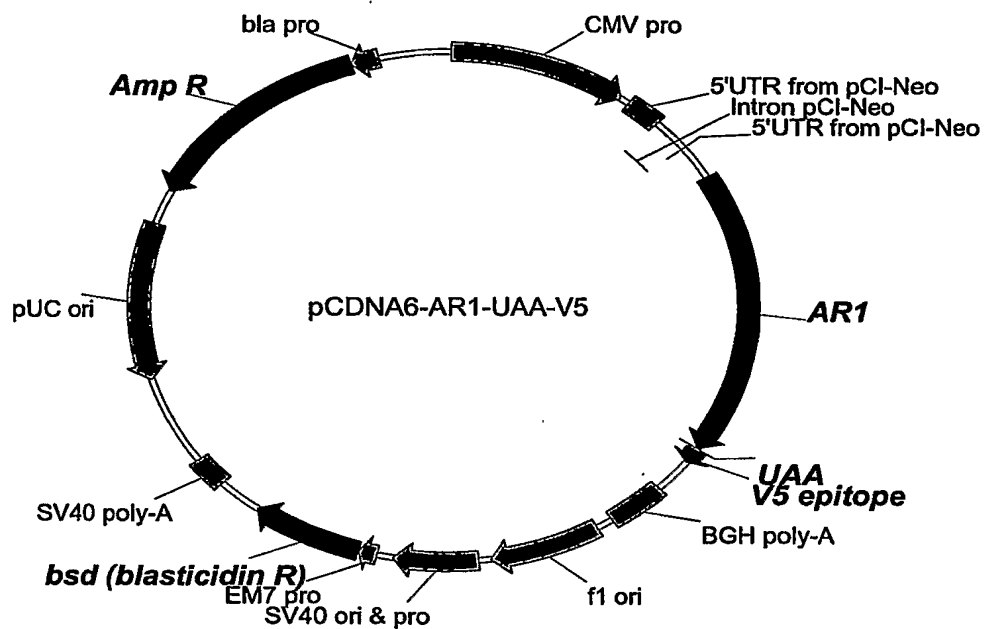
Histogram Statistics

Marker	Events	% Gated	Geo. Mean	CV
All	6850	100.00	7.85	103.35
% Pos. cells	2997	43.75	21.04	55.69
CV/Mean	6844	99.91	7.85	98.74

Figure 9



# Figure 10



# Figure 11

